Complexing of the CD-3 subunit by a monoclonal antibody activates a microtubule-associated protein 2 (MAP-2) serine kinase in Jurkat cells

Carine HANEKOM,* Andre NEL,* Cynthia GITTINGER,† Anelli RHEEDER* and Gary LANDRETH†‡

*Department of Internal Medicine, Stellenbosch University Medical School, P.O. Box 63, Tygerberg 7505, Cape Town, Republic of South Africa, and †Department of Neurology, Medical University of South Carolina, Charleston, South Carolina 29425, U.S.A.

Treatment of Jurkat T-cells with anti-CD-3 monoclonal antibodies resulted in the rapid and transient activation of a serine kinase which utilized the microtubule-associated protein, MAP-2, as a substrate *in vitro*. The kinase was also activated on treatment of Jurkat cells with phytohaemagglutinin, but with a different time course. The activation of the MAP-2 kinase by anti-CD-3 antibodies was dose-dependent, with maximal activity observed at concentrations of > 500 ng/ml. Normal human E-rosette-positive T-cells also exhibited induction of MAP-2 kinase activity during anti-CD-3 treatment. The enzyme was optimally active in the presence of 2 mM-Mn²⁺; lower levels of activity were observed with Mg²⁺, even at concentrations up to 20 mM. The kinase was partially purified by passage over DE-52 Sephacel with the activity eluting as a single peak at 0.25 M-NaCl. The molecular mass was estimated to be 45 kDa by gel filtration. The activation of the MAP-2 kinase was probably due to phosphorylation of this enzyme as treatment with alkaline phosphatase diminished its activity. These data demonstrate that the stimulation of T-cells through the CD-3 complex results in the activation of a novel serine kinase which may be critically involved in signal transduction in these cells.

INTRODUCTION

Activation of protein kinases following ligand binding appears to be critical for the transduction of signals from many cell surface receptors. These kinases may be generally divided into three groups according to their mechanism of induction after receptor occupancy. First, a number of receptors which are involved in growth and differentiation are themselves tyrosine-specific kinases (Hunter & Cooper, 1985). These include receptors for epidermal growth factor, platelet-derived growth factor, colony-stimulating factor, insulin-like growth factor-1 and insulin (Ushiro & Cohen, 1980; Ek *et al.*, 1982; Kasuga *et al.*, 1983; Hunter & Cooper, 1985). As a result of the activation of some of these kinases, the receptors may be autophosphorylated and this may, in turn, result in a change in receptor function (Cochet *et al.*, 1984; Iwashita & Fox, 1984; Sibley *et al.*, 1985; Patel *et al.*, 1987; Nel *et al.*, 1987a). Second, another group of receptors utilizes classical second messengers such as cyclic AMP, Ca²⁺, inositol phospholipids and calmodulin to activate protein kinase A, protein kinase C (PKC) and calmodulin-dependent kinases (Cheung, 1982; Gilman, 1984; Nishizuka, 1984). The existence of a third group of phosphotransferases has recently been suggested, namely kinases which are linked into complex amplification pathways along which their activities may be up- (or down-) regulated by other kinases (Hunter, 1987; Czech *et al.*, 1988). It is conceivable that the enzymes in the second group may act proximally to initiate such events.

The number of kinases in this putative category is potentially large and much remains to be learned about them (Hunter, 1987).

We have described a serine-threonine kinase which is rapidly and transiently activated by nerve growth factor in PC12 cells and epidermal growth factor in A431 cells (Landreth & Rieser, 1985; Landreth & Williams, 1987; G. Landreth, D. Smith, C. Gittinger & C. McCabe, unpublished work). This kinase has the ability to phosphorylate the microtubule-associated protein MAP-2, and a PC12 cell 250 kDa cytoskeletal protein, pp250, *in vitro*. This kinase is one of a small list of serine-threonine kinases with a preference for Mn²⁺ under phosphorylation conditions *in vitro* (Klemm & Elias, 1987; Yu *et al.*, 1987).

The identification of a MAP-2 kinase in PC12 and A431 cells as well as a similar activity in insulin-treated 3T3-L1 cells (Ray & Sturgill, 1987) suggests that this enzyme may be widely distributed and responsive to a number of different stimuli. Since one of our laboratories is involved in studying the biochemical events of T-cell activation, we were interested to see whether this kinase is present in T-cells and possibly involved in signal transduction during ligation of the T-cell antigen receptor (TCR)–CD-3 complex. The Jurkat T-cell line was chosen for the similarity of its responses to normal resting human T-cells during treatment with anti-CD-3 or anti-TCR monoclonal antibodies (mAbs) (Manger *et al.*, 1985). It is also useful for studying regulation of a variety of kinase activities involved in the T-cell response (Nel

Abbreviations used: PKC, protein kinase C; MAP, microtubule-associated protein; TCR, T-cell receptor; mAb, monoclonal antibody; PHA, phytohaemagglutinin; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine hydrochloride; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

‡ To whom correspondence should be addressed.

et al., 1987a, 1988). We report here that the anti-CD-3 treatment of Jurkat cells activates a protein kinase which appears similar to that observed in hormone-treated PC12, A431 and 3T3-L1 cells.

MATERIALS AND METHODS

Materials

Phytohaemagglutinin (PHA-P, purified grade), DEAE-Sephacel and alkaline phosphatase-Sepharose were from Sigma (St. Louis, MO, U.S.A.). OKT-3, OKT-4 and OKT-11 mAbs were from Ortho Pharmaceutical Corp. (Raritan, NJ, U.S.A.). A monoclonal anti-(T-cell antigen receptor) antibody (WT-31) was obtained from Becton Dickinson (Mountain View, CA, U.S.A.). H-7 [1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride] was obtained from Seikagaku Kogyo Co. (Tokyo, Japan). The IP-20 peptide was a gift from Dr. A. Nairn and R. Nichols (Rockefeller University, New York, NY, U.S.A.). Fractogel TSK55F gel filtration resin was from Merck, Inc. Fetal calf serum and other tissue culture reagents were from Gibco (Grand Island, NY, U.S.A.). All other chemicals were purchased from Sigma. Taxol was provided by the Drug Synthesis and Chemistry Branch, NCI.

Cell culture and stimulation

Subcloned populations of the Jurkat cell line, with both positive as well as negative fluorescence for the CD-3 (OKT-3) and TCR (WT-31) surface markers, as detected by flow cytometry, were grown in continuous culture in RPMI 1640 containing 10% fetal calf serum in an atmosphere of 5% CO₂ in air. Normal human T-cells were obtained from the peripheral blood of healthy adult volunteers by E-rosetting and density centrifugation as previously described (Pellegrino *et al.*, 1975).

Cells were resuspended at 3×10^6 cells/ml in RPMI 1640 before addition of the following stimuli for 0–20 min at 37 °C: anti-CD-3 (OKT-3 mAb), anti-TCR (WT-31 mAb), anti-CD-2 (OKT-11 mAb), anti-CD-4 (OKT-4 mAb) and PHA. Control cultures received no further additions.

Preparation of cellular lysates

Control of stimulated cells were briefly centrifuged in an Eppendorf microfuge and resuspended in lysis buffer (50 µl/10⁶ cells) containing 20 mM-Tris, pH 7.4, 1 mM-EGTA, 1 mM-phenylmethanesulphonyl fluoride, 50 µM-sodium vanadate, 50 mM-sodium fluoride and 20 mM-*p*-nitrophenylphosphate. The cells were disrupted by sonication (2 × 5 s) and centrifuged in a microfuge for 15 min at 4 °C. The supernatants were collected and combined with 100 µl of DEAE-Sephacel, previously equilibrated in the same buffer. The sample was incubated with the resin for 5 min at 4 °C, whereupon the unbound material was removed through centrifugation of the slurry. The resin was washed three times in 1 ml of lysis buffer containing 0.05 M-NaCl, and the enzyme was then eluted from the resin by addition of lysis buffer containing 0.2 M-NaCl.

Phosphorylation assays

Protein kinase activity was assayed by adding 5 µg of protein of DEAE eluate to reaction vials containing lysis buffer plus 1 mM-MnCl₂ and 5–10 µg of MAP-2 or 10 µg

of pp250 preparation (Landreth & Williams, 1987). Phosphorylation was initiated by addition of 10 µM-[α -³²P]ATP (2–8 µCi/nmol). The reaction was carried for 20 min at 4 °C and stopped by addition of electrophoresis sample buffer and boiling for 5 min. The samples were separated by electrophoresis on SDS/polyacrylamide (3–13%) gels (Laemmli, 1970). Autoradiographs of the stained and dried gels were prepared by exposure to Kodak XAR-5 film. The MAP-2 bands were excised from the gel and ³²P incorporation into MAP-2 was determined by Čerenkov counting.

Ion exchange and gel filtration chromatography

Cellular extracts obtained from 10⁸ anti-CD-3-treated Jurkat cells were applied to a 1 cm × 2 cm DE-52 column equilibrated in 20 mM-Tris, pH 7.4/1 mM-EGTA. The column was washed with the same buffer containing a 0.05 M-NaCl and then developed with a linear 0.05–0.6 M-NaCl gradient at a flow rate of 1 ml/min with 0.5 ml fractions collected. Aliquots (10 µl) from each fraction were assayed in duplicate for MAP-2 kinase activity.

Peak fractions obtained from the DE-52 column were pooled and applied to a 1 cm × 30 cm TSK HW 55F gel filtration column in 200 mM-Tris, pH 7.4/10 mM-EGTA. The enzyme was eluted at a flow rate of 30 ml/h and 0.5 ml fractions were collected. Fractions (40 µl) were assayed for MAP-2 kinase activity as described above. The column was calibrated using bovine serum albumin, ovalbumin and α -chymotrypsin.

Preparation of MAP-2 and pp250 substrates

MAPs were prepared from rat brains by the method of Vallee (1982). The MAP preparation was enriched for the heat-stable 280 kDa MAP-2 by heating for 5 min at 100 °C, and the precipitated proteins were removed by centrifugation (Kim *et al.*, 1979).

The PC12 cell substrate pp250 was prepared through heat treatment of cytoskeletal extracts (Landreth & Williams, 1987).

Phosphoamino acid analysis

Radiolabelled MAP-2 was excised from dried gels and phosphoamino acid analysis performed exactly as described by Cooper *et al.* (1983).

Alkaline phosphatase treatment of MAP-2 kinase

The kinase preparation (5 µg of protein) obtained following batch absorption to DEAE-Sephacel was incubated in 50 µl of phosphorylation buffer with 1 unit of alkaline phosphatase-Sepharose for 15 min at 22 °C. The resin was removed by centrifugation and MAP-2 kinase activity assayed as described above.

RESULTS

Anti-CD-3 treatment enhances MAP-2 kinase activity

Treatment of Jurkat cells with an anti-CD3 mAb (OKT-3) resulted in the activation of a protein kinase which phosphorylated the 280 kDa microtubule-associated protein MAP-2 (Fig. 1). The activity was increased by approx. 5-fold after 5 min of treatment with OKT-3 over that observed in control cells (Fig. 1). The magnitude of stimulation varied from 2–7-fold (mean 3.5) in six separate experiments. The anti-CD-3-activated kinase in Jurkat cells could also phosphorylate pp250, a cyto-

Fig. 1. Anti-CD-3 treatment of Jurkat cells stimulates the phosphorylation of MAP-2 and pp250 *in vitro*

Jurkat cells were treated with anti-CD-3 monoclonal antibody for 5 min at 37 °C, and the enzyme partially purified by batch absorption to DE-52 as described in the text. The enzyme preparation was combined with 10 µg of MAP-2 or pp250 substrate and the phosphorylation reaction carried out. The labelled proteins were separated on SDS/polyacrylamide-gel electrophoresis (4–12 % gels). An autoradiogram is shown with the positions of molecular mass markers (kDa) and the substrates as indicated. Each reaction was run in duplicate.

Table 1. Comparison of mAb and PHA in stimulating MAP-2 kinase activity in CD-3⁺ cells

Jurkat cells were incubated with the indicated agents for 5 min at 37 °C, and MAP-2 kinase activity was measured as described in the Materials and methods section. 5 µg of MAP-2 were used per assay. The data represent means ± S.D. from triplicate determinations.

Lectin or mAb	Receptor	³² P incorporation (c.p.m.)
None (control)	–	930 ± 121
OKT-3 (1 µg/ml)	CD-3	2534 ± 132
WT-31 (0.5 µg/ml)	TCR	2070 ± 315
OKT-4 (1 µg/ml)	CD-4	588 ± 0
OKT-11 (1 µg/ml)	CD-2	812 ± 82
PHA-P (10 µg/ml)	?CD-3/CD-2	2350 ± 178

skeletally-associated protein found in PC12 cells. We have shown that MAP-2 and pp250 are alternative substrates for the same enzyme in PC12 cells (G. Landreth, D. Smith, C. Gittinger & C. McCabe, unpublished work).

The specificity of the response was demonstrated by the failure of monoclonal antibodies to the CD-2 receptor (OKT-11) or CD-4 receptor (OKT-4) to stimulate the kinase activity (Table 1). Moreover, anti-CD-3 mAb failed to stimulate the kinase activity in a CD-3/TCR-negative Jurkat mutant: ³²P incorporation values were

Table 2. Comparison of MAP-2 kinase activation in Jurkat and E-rosette positive normal human T-cells

An enriched population of normal human T-cells was obtained by E-rosetting. The cells were incubated with the indicated agents for 5 min at 37 °C and MAP-2 kinase was assayed as described in the Materials and methods section. The data represent means ± S.D. from triplicate determinations

	³² P incorporation (c.p.m.)	
	Jurkat cells	Normal T-cells
Unstimulated control	482 ± 35	98 ± 5
OKT-3 (1 µg/ml)	2066 ± 141	187 ± 9
TPA (100 ng/ml)	2261 ± 152	373 ± 5

930 ± 121 and 944 ± 45 c.p.m. for control and anti-CD-3-treated populations respectively. Reaction of CD-3-positive Jurkat cells with WT-31, a mouse monoclonal against a framework determinant of the α/β T-cell antigen receptor, produced comparable increases in kinase activity (Table 1). The mitogenic lectin PHA was also an effective inducer of MAP-2 kinase activity (Table 1). Normal human E-rosette-positive T-cells also exhibited activation of a MAP-2 kinase during anti-CD-3 stimulation (Table 2). The mitogenic phorbol ester, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), a specific protein kinase C agonist, also stimulated MAP-2 kinase activity after 5 min of treatment (Table 2). Although MAP-2 may also serve as a substrate for PKC (Tsuyama *et al.*, 1986) it is unlikely that the TPA effect in this experiment was directly mediated by this enzyme as reactions were run in the absence of Ca²⁺, phosphatidylserine and diolelin (also see below).

Time- and dose-dependence of induction of MAP-2 kinase activity

The induction of MAP-2 kinase activity in Jurkat cells by anti-CD-3 is a rapid and transient event; maximal stimulation was observed within 2–5 min after initiating anti-CD-3 treatment, whereupon the activity declined rapidly in the presence of the ligand (Fig. 2a). As expected, treatment with WT-31, an mAb which reacts with a different site on the TCR–CD-3 complex, yielded identical results (results not shown). PHA also activated the kinase (Table 1); however, the response proceeded with different kinetics, with maximal activity observed at approx. 30 min followed by a slow decline after this (Fig. 2b). The evanescent response to anti-CD-3 treatment is similar to that which was obtained during hormone treatment of PC12 and A431 cells (G. Landreth, D. Smith, C. Gittinger & C. McCabe, unpublished work) and is similar to that observed by Ray & Sturgill (1987, 1988b) on insulin treatment of 3T3-L1 cells.

Activation by anti-CD-3 mAb was dose-dependent with maximal activity observed at a concentration of 500 ng/ml (Fig. 3). This is in agreement with the antibody range required for induction of interleukin-2 responsiveness in normal humans T-cells during stimulation with the F(ab')₂ fragments of OKT-3 (Tsoukas *et al.*, 1985).

Fig. 2. Time course of anti-CD-3 and PHA-dependent activation of MAP-2 kinase

Jurkat cells were incubated with 1 μ g of anti-CD-3/ml (a) or 1 μ g of PHA/ml (b) for the indicated periods and MAP-2 kinase activity determined. The data represent the means $\pm$ S.D. from triplicate determinations.

Partial purification of MAP-2 kinase activity

The anti-CD-3-activated MAP-2 kinase activity was resolved by passage over a DE-52 Sephacel column and the activity was eluted as a single peak at 0.25 M-NaCl (Fig. 4). Extracts from untreated control cells exhibited only low levels of kinase activity. The behaviour of the activity on anion-exchange chromatography was identical to that previously observed in both PC12 and A431 cells (G. Landreth, D. Smith, C. Gittinger & C. McCabe, unpublished work).

Gel filtration of peak fractions obtained from DEAE columns resulted in the elution of a single symmetric peak of activity with an apparent molecular mass of 42–45 kDa (Fig. 5).

Cation-dependence of MAP-2 kinase activity

The enzyme displayed an unusual dependence on Mn^{2+} , with maximum activity observed at 2 mM but diminishing activity at higher concentrations (Fig. 6). The MAP-2 kinase exhibited much lower levels of activity in the presence of Mg^{2+} . The Mg^{2+} -dependent phosphorylation was only approximately half that observed with Mn^{2+} even at concentrations of 10–20 mM. The cation concentration-dependence is similar to that

Fig. 3. Anti-CD-3 dose-dependent activation of MAP-2 kinase

Jurkat cells were incubated with the indicated concentrations of anti-CD-3 monoclonal antibody for 5 min at 37 °C, and MAP-2 kinase activity was determined. The data represent the means $\pm$ S.D. from triplicate determinations.

Fig. 4. Ion-exchange chromatography of MAP-2 kinase

Jurkat cells were incubated for 5 min in the absence or presence of 1 μ g of anti-CD-3/ml. Cellular lysates were prepared and applied to a DEAE-Sephacel column in 20 mM-Tris, pH 7.4/1 mM-EGTA, the column was washed in the same buffer containing 50 mM-NaCl and then developed with a 0.05–0.6 M-NaCl gradient. Aliquots (10 μ l) of the column fractions were assayed for MAP-2 kinase activity.

previously observed with MAP-2 kinase isolated from PC12 and A431 cells (G. Landreth, D. Smith, C. Gittinger & C. McCabe, unpublished work). The preference for Mn^{2+} is uncharacteristic of a serine-threonine kinase, but is more typical of tyrosine-specific kinase activity (Hunter & Cooper, 1985).

MAP-2 is phosphorylated on serine residues

Phosphoamino acid analysis of radiolabelled MAP-2 revealed serine as the only labelled amino acid (Fig. 7), and therefore distinguished the kinase under study from tyrosine kinases which are also able to phosphorylate this substrate (Kadowaki *et al.*, 1985; Nishida *et al.*, 1987).

Fig. 5. Gel filtration chromatography of MAP-2 kinase

Peak fraction obtained from the DEAE-Sephacel column were collected, pooled, concentrated and applied to a TSK HW55F gel filtration column. The column was eluted with 20 mM-Tris, pH 7.4/1 mM-EGTA at a flow rate of 1 ml/min, and 0.5 ml fractions were collected. Aliquots (40 μ l) of the fractions were assayed for MAP-2 kinase activity. The column was calibrated using bovine serum albumin (BSA), ovalbumin (Oval) and α -chymotrypsin.

Fig. 6. Cation dependence of MAP-2 kinase activity

Jurkat cells were treated for 5 min with 1 μ g of anti-CD-3/ml and the kinase was partially purified by batch absorption to DEAE-Sephacel followed by gel filtration. The partially purified kinase preparation was combined with MAP-2 substrate (10 μ g) and radiolabelled ATP with the indicated concentrations of Mn^{2+} ($\square$) or Mg^{2+} ($\blacklozenge$) and the phosphorylation reaction carried out. The data represent the means $\pm$ S.D. from triplicate determinations.

MAP-2 kinase is distinct from other kinases involved in T-cell activation

It was important to verify that the kinase activity under study was distinct from well-known kinases which are involved in the T-cell response (Cheung *et al.*, 1983;

Fig. 7. Phosphoamino acid analysis of MAP-2

Radiolabelled MAP-2 was prepared by incubation of 10 μ g of the substrate with the kinase preparation obtained from anti-CD-3-treated Jurkat cells and [32 P]ATP. The labelled band was excised from the gel and hydrolysed. The labelled phosphoamino acids were separated by electrophoresis at pH 3.5. Shown is an autoradiogram with the position of the phosphoamino acid standards indicated.

Fig. 8. Effect of protein kinase inhibitors on MAP-2 kinase activity

Jurkat cells were incubated in the absence or presence of 1 μ g of PHA/ml for 30 min at 37 $^{\circ}$ C. The enzyme was added to the standard reaction mixture (control) or containing IP-20 peptide, a specific inhibitor of A-kinase, 50 μ M-trifluoperazine (TFP) or 20 μ M-H-7. The reaction was carried out for 20 min at 4 $^{\circ}$ C, the proteins were separated by SDS/polyacrylamide-gel electrophoresis and autoradiograms were prepared. The radioactivity incorporated into MAP-2 was measured by densitometrically scanning the autoradiogram. The data are the means of duplicate determinations and are expressed in arbitrary units. Control, solid bars; anti-CD-3-treated, hatched bars.

Patel *et al.*, 1987; Nel *et al.*, 1987a,b, 1988), some of which are also known to phosphorylate MAP-2; these include PKC, cyclic AMP-dependent kinases and calmodulin-dependent kinases (Fukunaga *et al.*, 1982;

Table 3. Reversible activation of MAP-2 kinase

Jurkat cells were incubated for 5 min at 37 °C in the absence or presence of 1 µg of anti-CD-3/ml. The cells were lysed in 20 mM-Tris, pH 7.4/1 mM-EGTA with or without the addition of phosphatase inhibitors (20 mM-*p*-nitrophenylphosphate, 50 mM-NaF and 50 µM-sodium vanadate). The kinase was partially purified by batch absorption to DEAE-Sephacel and assayed for MAP-2 kinase activity. Aliquots (5 µg of protein) of the anti-CD-3-treated samples were then combined with 1 unit of alkaline-phosphatase-Sepharose and incubated for 10 min at 22 °C. The resin was removed by centrifugation and MAP-2 kinase activity determined. The data represent means ± S.D. from triplicate determinations.

	[³² P]MAP-2 (c.p.m.)	
	+ Phosphatase inhibitors	– Phosphatase inhibitors
Control	1256 ± 48	493 ± 12
Anti-CD-3	2386 ± 334	406 ± 6
Anti-CD-3 + AP	55 ± 5	42 ± 2

Yamauchi & Fujisawa, 1982; Theurkauf & Vallee, 1983*a,b*; Kadowaki *et al.*, 1985; Tsuyama *et al.*, 1986; Nishida *et al.*, 1987). MAP-2 kinase was partially purified by batch absorption and elution from DEAE-Sephacel beads, and incubated with MAP-2 in the presence of specific kinase inhibitors (Fig. 8). IP-20, a peptide identical to the active site of the Walsh inhibitor which specifically inhibits A-kinase (Cheung *et al.*, 1986), had no effect on the activated kinase *in vitro*. Moreover, MAP-2 kinase activity was not increased in extracts from Jurkat cells which had been previously treated with dibutyryl cyclic AMP (results not shown). Similarly, trifluoperazine, an inhibitor of PKC and calcium/calmodulin-dependent kinases (Kuo *et al.*, 1984) did not significantly inhibit the MAP-2 kinase activity. We observed only a slight inhibition of the MAP-2 kinase activity in the presence of H-7, a potent inhibitor of both A and C-kinases (Hidaka *et al.*, 1984). It should be noted that the assay buffer contained 1 mM-EGTA, which should effectively suppress calcium-dependent kinase activities.

Reversible activation of the MAP-2 kinase

The magnitude of the stimulation of MAP-2 kinase was found to be somewhat variable, and optimal recovery of kinase activity was achieved only when the enzyme was isolated in the presence of phosphatase inhibitors. This observation suggested that the active form of the enzyme was phosphorylated (Table 3). This was tested directly by incubation of the partially purified enzyme with alkaline phosphatase. Treatment of the enzyme with alkaline phosphatase resulted in the loss of MAP-2 kinase activity. This is consistent with previous observation on the MAP-2 kinase activity isolated from PC12 cells (G. Landreth, D. Smith, C. Gittinger & C. McCabe, unpublished work) and a similar activity present in 3T3-L1 cells (Ray & Sturgill, 1987, 1988*a,b*). Interestingly, phosphatase treatment resulted in reduction of activity well below that found in untreated control cell extracts.

DISCUSSION

The present data demonstrate that anti-CD-3 treatment of Jurkat cells rapidly activates a 45 kDa Mn²⁺-preferring serine kinase which utilizes MAP-2 and pp250 as substrates. The activity reported here is quite similar to that found in PC12 cells and A431 cells as shown by the following characteristics: chromatographic behaviour, cation dependence, substrate specificity and molecular mass (Landreth & Rieser, 1985; Landreth & Williams, 1987; G. Landreth, D. Smith, C. Gittinger & C. McCabe, unpublished work). Significantly, the time course of activation of this kinase in lymphocytes is similar to that of nerve-growth-factor-treated PC12 cells and epidermal growth factor-treated A431 cells (Landreth & Rieser, 1985; Landreth & Williams, 1987; G. Landreth, D. Smith, C. Gittinger & C. McCabe, unpublished work).

Ray & Sturgill (1987, 1988*a,b*) have independently identified a MAP-2 kinase in 3T3-L1 cells which was activated following insulin treatment. This enzyme is similar in many respects to the activity reported here. Interestingly, the 3T3-L1 enzyme itself is phosphorylated on tyrosine residues, suggesting that its activation results from phosphorylation by the insulin receptor kinase (Ray & Sturgill, 1988*a*).

MAP-2 is a 280 kDa microtubule-associated protein found within the cell body and dendrites of neurons (Olmsted, 1986). Its interaction with other cytoskeletal elements is regulated by phosphorylation. Although it has not been reported to be found in T-cells, it is of course possible that related substrates with similar functions may exist.

Although MAP-2 may be phosphorylated by a number of different kinases (Fukunaga *et al.*, 1982; Yamauchi & Fujisawa, 1982; Theurkauf & Vallee, 1983*a,b*; Kadowaki *et al.*, 1985; Tsuyama *et al.*, 1986; Nishida *et al.*, 1987), the particular kinase under study is probably novel for the following reasons. First, it is a Mn²⁺-preferring serine kinase, a property usually associated with tyrosine kinases (Hunter & Cooper, 1985). Two examples of a Mn²⁺-dependent serine kinase have been reported, namely Kemptide kinase and protein kinase P, a phospholipid-stimulated kinase (Yu *et al.*, 1987; Klemm & Elias, 1987). Secondly, we have been able to distinguish this kinase from well-known kinases in CD-3-TCR mediated T-cell responses (Patel *et al.*, 1987; Nel *et al.*, 1987*a*, 1988). PKC activity was excluded by virtue of its higher molecular mass, its requirement for allosteric cofactors during activation, and almost total inhibition of its activity by 20 µM-H-7. Because this isoquinoline agent acts at the active site of the enzyme, it may be assumed that phosphorylation by M-kinase, a 50 kDa proteolytic product of PKC, can also be excluded (Hidaka *et al.*, 1984). Cyclic AMP-dependent kinase involvement was ruled out through use of a specific inhibitor as well as intact cell stimulation studies. The potential role of calcium/calmodulin-dependent kinases was excluded by lack of effect of trifluoperazine at doses which are inhibitory to calmodulin or intact T-cell responses (Cheung *et al.*, 1983) and its activity in the absence of Ca²⁺.

Although there are a number of surface receptors which may initiate T-cell activation, the classical antigen-dependent pathway relies on physical interaction between the α/β-TCR with appropriately presented antigen (reviewed by Weiss & Imboden, 1987). This disulphide-

linked heterodimer is intimately but non-covalently associated with the CD-3 complex, which in turn comprises at least four heterologous subunits (Petty *et al.*, 1987). These subunits act in an unknown fashion to introduce a number of intracellular second messengers (Valentine *et al.*, 1985; Weiss & Imboden, 1987). The most well defined involves the activation of phospholipase C which leads to generation of two other second messengers, 1,2-diacylglycerol and inositol 1,4,5-trisphosphate, through the hydrolysis of phosphatidylinositol 4,5-bisphosphate (Weiss & Imboden, 1987). 1,2-Diacylglycerol is the endogenous activator of PKC, whereas inositol 1,4,5-trisphosphate promotes the release of Ca^{2+} from intracellular stores. Antibodies as well as certain mitogenic lectins which ligate the TCR-CD-3 complex may activate similar events (Nel *et al.*, 1987a, 1988).

Apart from the inositol phospholipid/PKC pathway there are other second messenger systems and kinases which are involved in the T-cell response (Patel *et al.*, 1987; Nel *et al.*, 1988). Our study introduces another kinase to this growing list. The precise way in which MAP-2 kinase activity is induced is still uncertain. What is clear, however, is that its activity is reversibly up- and down-regulated following binding of ligand to the TCR-CD-3 complex. This could involve a phosphorylation/dephosphorylation cycle as previously suggested in PC-12 cells (G. Landreth, D. Smith, C. Gittinger & C. McCabe, unpublished work) and 3T3-L1 cells (Ray & Sturgill, 1988a; Sturgill *et al.*, 1988). It is therefore interesting to note that we have found that this kinase may also be activated during treatment with TPA, a specific PKC-agonist (Table 2). It is possible that induction of MAP-2 activity may be PKC-dependent, in which case this kinase could be classified amongst those kinases which are linked-in-series for the purpose of signal modification (Hunter, 1987; Sturgill *et al.*, 1988). This aspect is currently under study.

Although ligation of TCR-CD-3 by monoclonal antibodies is able to elicit biochemical T-cell responses, signalling is incomplete in terms of intact cell responses if the ligating antibodies are added in soluble form (Nel *et al.*, 1987a; Weiss & Imboden, 1987). Immobilization of the stimulating mAb on a bead or to macrophage Fc receptors provides a more complete signal (Valentine *et al.*, 1985). Since we used soluble mAbs, it would seem as if induction of MAP-2 kinase activity is primarily dependent on receptor occupation. It is however possible that aggregation of CD-3 may enhance activity even further. It is also likely that stoichiometry of the TCR may influence cellular signal generation. For instance, when a more complex ligand such as PHA was introduced, a different MAP-2 kinase stimulation response was obtained (Fig. 2b). PHA interacts with CD-3 as well as with a number of unidentified surface glycoproteins (Valentine *et al.*, 1985; O'Flynn *et al.*, 1986) and may therefore create complex associations of TCR with other cell surface proteins. Even though this may not be the complete explanation, these findings show that there are fundamental differences between stimulation with monoclonal antibodies and PHA in spite of this lectin's ability to interact with the TCR-CD-3 receptor (Valentine *et al.*, 1985; O'Flynn *et al.*, 1986).

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